Antigen Handling in Antigen-Induced Arthritis in Mice

An Autoradiographic and Immunofluorescence Study Using Whole Joint Sections

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Antigen localization after intraarticular antigen injection was studied in immune and nonimmune mice using autoradiographic and immunofluoresence techniques on whole joint sections. After intraarticular injection of radiolabeled methylated bovine serum albumin (^{125}I-mBSA) in immune mice, labeling in the synovium and synovial exudate diminished rapidly, apart from some deposits in fibrinlike material present in the joint cavity. Long-term antigen retention was found in avascular and hypovascular structures lining the joint cavity, albeit not along the whole surface; eg, labeling remained present at the edges of the femoral condyle hyaline cartilage but not at the central weight-bearing region; long-term retention at ligaments was only found at the insertion sites. Immunofluorescence data in immune animals showed antigen retention together with the presence of immunoglobulins and complement, indicating that antigen is retained at least in part in the form of immune complexes. Nonimmune mice showed even higher long-term antigen retention than immune animals, probably related to physicochemical properties of the antigen enabling nonimmune binding to articular structures, but also indicating that the presence of joint inflammation in the immune animals enhances antigen clearance. Histologic examination of the ligaments and patellar cartilage of immune mice did reveal that long-term antigen retention was not anatomically related to nearby inflammation or to local tissue damage. The importance of long-term antigen retention for the chronicity of arthritis may lie in the leakage of small amounts of this antigen to joint compartments where it does behave as an inflammatory stimulus; it may further be that it renders the joint a specifically hypersensitive area. (Am J Pathol 1982, 108:9–16)

ANTIGEN-INDUCED ARTHRITIS (AIA) has been widely used as a model for the study of human rheumatoid arthritis in view of its similar histopathologic character and chronicity.[1–4] Studies on antigen handling in rabbit knee joints have indicated that a small part of the arthritis-inducing intraarticularly injected antigen (IA antigen) is retained in the joint for at least several months,[5–7] and it has been suggested that the chronicity of AIA is caused by persistence of IA antigen.[5,6] Studies on dissected joint structures have shown that retention of antigen, presumably in the form of immune complexes, takes place predominantly in the superficial layers of hyaline cartilage, menisci, and ligaments, but not in the synovium.[6] Since in these studies the joint structures had to be dissected, they gave no information on the exact anatomic localization of the antigen within the intact joint, the subtle local differences in amount of antigen, and the relation between antigen localization and inflammation or tissue damage, at various times after arthritis induction.

The recent development of AIA in mice[8,9] has made it possible to investigate morphologically the fate of intraarticularly injected antigen, since it is

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technically possible to prepare autoradiographs[9] and immunofluorescence stains[10] of whole joint sections in these small animals. With these techniques we studied the fate of IA antigen in both immune and nonimmune mice. In addition, we investigated whether antigen injected into a joint with already ongoing AIA is handled differently from antigen after a first arthritis-inducing intraarticular injection.

Materials and Methods

Animals

Male C57B1 mice aged 6–8 weeks at the start of the immunization were used.

Iodination of Antigen

^{125}Iodine labeling of methylated bovine serum albumin (mBSA) was performed by the chloramine-T method.[11] ^{125}I-mBSA was separated from free ^{125}I by Sephadex G25 fractionation. The specific activity was approximately 2 μCi/μg.

Immunization and Arthritis Induction

Mice were immunized with 100 μg mBSA in 0.1 ml Freund's complete adjuvant emulsion on Days 0 and 7 as previously described.[9] On Day 21 knee joint arthritis was induced by intraarticular injection of 100 μg mBSA in 10 μl saline.

Experimental Design

Antigen elimination and retention was studied in three experimental groups: immune mice (I), nonimmune mice (II), and arthritic mice (III). Group I was immunized with mBSA as described above, and arthritis was induced in both knee joints by intraarticular injection of 100 μg ^{125}I-mBSA (1 μCi). Group II consisted of nonimmune mice, aged 10–12 weeks, which likewise received 100 μg ^{125}I-mBSA (1 μCi) in both knee joints. Group III consisted of arthritic mice. If antigen handling by the joint is an important determinant of the course of chronic arthritis, it would be relevant to know how IA antigen is handled, not only after a first arthritis-inducing injection but also after a second injection into an already chronically inflamed joint. In this group (III) unilateral arthritis was induced by intraarticular injection of 100 μg mBSA into the right knee joint 21 days after immunization. Four weeks later both knee joints were given injections of 100 μg ^{125}I-mBSA (1 μCi). This protocol ensured that possible differences between antigen handling after a first, as compared with a second, injection could not be due to changed systemic immunity after the first injection and therefore had to be related to local factors in the already inflamed joint.

At various days (0–28 as indicated in the Results section) after intraarticular injection of radiolabeled antigen groups of mice were killed by ether anesthesia. The knee joints were removed in toto and fixed in 10% phosphate-buffered formalin. Total tissue radioactivity of the knee was counted in a scintillation counter of the well type and expressed as percentage of the initial count rate immediately after antigen injection. Values at various days were corrected for physical decay. Thereafter, the tissues were processed for histologic study and autoradiography.[9]

Antigen Localization by Autoradiography

Total knee sections (6 μ) were prepared and mounted on gelatin-coated slides. These were dipped in K_5 emulsion (Ilford, Basildon, Essex, England) and exposed for 1–6 weeks. After this period the slides were developed and stained with hematoxylin and eosin (H&E).

Immunofluorescence

Another series of mice as described in Groups I and II under Experimental Design were given injections of unlabeled mBSA into both knee joints, and series of 3 mice were killed at various days after IA antigen injection. The knee joints were removed *in toto* and rapidly frozen to the wall of a test tube by immersion in liquid nitrogen. Six-micron sections of the undecalcified whole knee joints were prepared as described before.[10] The sections were analyzed for the presence of IgG, IgM, and complement, with the use of the following fluorescein-conjugated antisera: goat anti-mouse IgG and goat anti-mouse IgM (Meloy Laboratories, Springfield, Va), Goat anti-mouse C_3 (USB Cleveland, Ohio). Detection of antigen was performed with an Ig fraction of a rabbit anti-mBSA antiserum, prepared in rabbits by immunization with 1 mg mBSA in Freund's complete adjuvant, followed by boosting two times with 1 mg mBSA in Freund's incomplete adjuvant. Swine anti-rabbit Ig-FITC (Cappel Laboratories, Cochranville, Pa) was used as a second layer.

Appropriate controls included exposure of the sections to nonimmune sera, omission of the first layer, and analysis of noninjected control joints. As a positive control for complement staining, arthritic joints isolated 2 days after zymosan injection were used, since zymosan is known to be a potent complement activator. These joints were also used as negative but inflamed controls for the specificity of the fluorescence found with the anti-mBSA serum in the mBSA-induced arthritic joints. No staining was found with this antiserum in the zymosan joints.

Results

Autoradiographic Studies

Immune and nonimune mice were injected intraarticularly with ^{125}I-labeled mBSA and autoradiographs of whole joint sections were prepared at various days after injection. In immune animals, shortly after injection (2 hours) the antigen was localized predominantly in the synovial cavity, was found along the whole surface of its lining structures, such as hyaline cartilage, menisci, ligaments, and synovial lining cells, but was not found in the subsynovial tissue. At Day 2 pronounced exudation in the joint space and infiltration of the synovium was seen. Antigen could only be sparsely detected in the subsynovial tissue but was clearly present in the exudate, in the synovial lining cells, and at the surface of avascular and hypovascular structures (Table 1). At Day 4 labeling in the exudate diminished clearly, except for some deposits in fibrinlike material, localized mostly in relatively cell-free areas (Figure 1). From Day 7 on, antigen was found only sporadically in the exudate but remained in the avascular and hypovascular structures, albeit not always along the whole surface. Labeling at meniscal cartilage, for example, seemed to be more intense in late frontal semiserial sections showing small menisci with fibrocartilage than in superficial sections showing meniscal cartilage with a more hyalinelike appearance (Figure 2).

In the nonimmune animals slight signs of inflammation developed at Day 2, with some exudate cells in the synovial cavity. Localization of antigen at that time was not different from that seen in the immune animals. At Day 7 and later on, signs of inflammation were no longer present. At Day 7 antigen was

Table 1 – Antigen Localization at Various Days After Intraarticular Injection of ^{125}I-mBSA in Immune Mice

	Days after arthritis induction					
	2	4	7	10	14	28
Synovial cavity	++*	+	±	–	–	–
Synovial lining cells	+	+	–	–	–	–
Subsynovial tissue	±	±	–	–	–	–
Hyaline cartilage and menisci	+	+	+	+	+	+
Ligaments	+	+	±	±	±	±

* Relative score of antigen localization, only to compare relative labeling of different areas at one particular day. Values are based on examination of autoradiographs of 6 knee joints for each time interval.

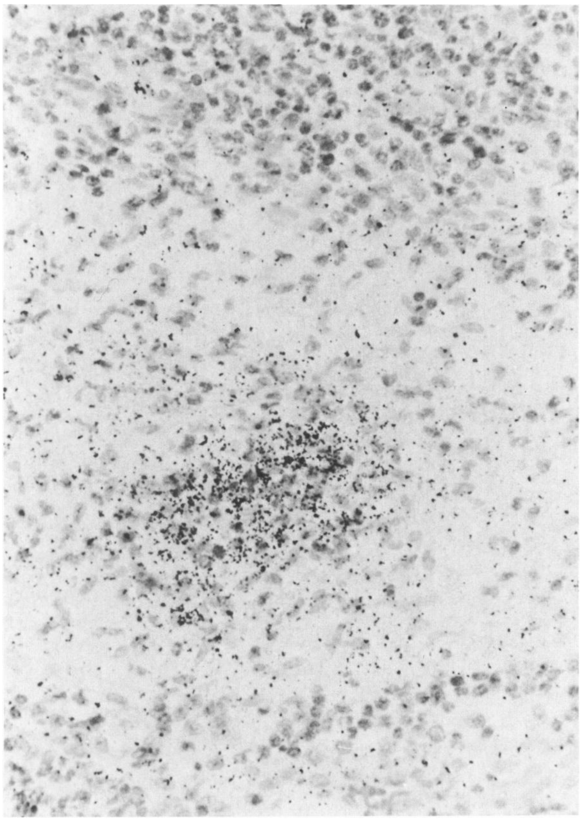

Figure 1 – Autoradiograph of synovial exudate at Day 4 after intraarticular injection of ^{125}I-mBSA in immune mice. Labeling represents antigen trapped in fibrinlike material, which is relatively cell-free. (H&E, ×206)

clearly located at the surface of avascular and hypovascular structures but was also still present at the synovial lining cells (Figure 3). The extent and intensity of the labeling in the nonimmune animals exceeded that seen in the immune mice, especially at sites showing overgrowth with granulation tissue in the latter animals, eg, at ligaments and marginal zones of cartilage structures. This difference between immune and nonimmune animals remained present until the end of the period studied and was demonstrated once more by external radioactivity measurements of dissected knee joints (Figure 4).

Immunofluorescence

Immunofluorescence was performed with a rabbit anti-mBSA antiserum on fresh frozen tissue sections. Additional sections were stained for the presence of immunoglobulins and complement. In immune animals the localization of mBSA followed the patterns found with autoradiography. The immunofluorescence technique proved to be less sensitive than autoradiography, for after Day 10 positive cartilage

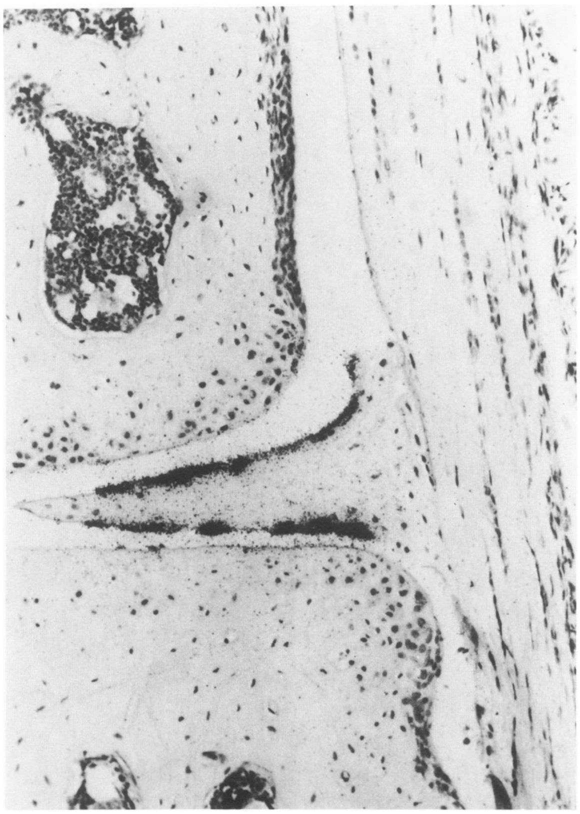

Figure 2—Autoradiograph at Day 28 after intraarticular injection of ^{125}I-mBSA in immune mice. Note the persistent labeling at the fibrocartilage of the meniscus. (H&E, ×83)

staining for mBSA was found only sporadically. IgM staining was strong in the exudate and in the cartilage surface from Day 2 and remained positive in the cartilage until Day 28. IgG was present in the cartilage surface at Day 2, increasing in intensity until Day 7 and remaining high until the end of the period studied. Complement staining was faint but unequivocally present in the cartilage and followed the patterns found for antigen and immunoglobulins, indicating the presence of immune complexes. In contrast to the immune animals, in the nonimmune animals antigen was detectable until Day 28, reflecting the higher retention of antigen in the latter group (Figure 4). IgM and IgG staining was faint, not exceeding the staining found for control cartilage of uninjected mice. Complement staining was always negative.

Handling of IA Antigen Injected Into Chronically Inflamed Joints

Figure 5 shows that antigen is more rapidly cleared after a second injection of antigen into the inflamed right knee joint than after a first injection of antigen into the contralateral knee joint of the same animal. Histologic sections showed more pronounced inflammation at Days 2 and 4 in the joint given two injections than in the contralateral joint. Thereafter, inflammatory scores were equal or even higher in the contralateral knee joints. Autoradiographs showed labeling patterns resembling those described for a first injection. However, the disappearance of activity from the synovial cavity was extremely rapid in the knee joint given two injections as compared with the contralateral knee joint given one injection. Moreover, the labeling of cartilage structures at Day 2 and accordingly at later stages seemed relatively low as compared with the contralateral joint. Finally, labeling at ligaments was already clearly decreased as compared with the contralateral knee at Day 2 due to the extremely rapid clearance from sites showing overgrowth with granulation tissue.

Long-Term Antigen Retention at Special Sites Within the Joint

As mentioned before, antigen remained in the avascular and hypovascular structures, but not always along the whole surface. There are sites of predilection for antigen persistence in the joint, which are consistently found in all experimental groups studied. Labeling is clearly present at the edges of the medial or lateral femoral condyles, as illustrated in Figure 6, but not at the central weight-bearing region. Variable labeling was found in frontal semiserial sections taken from the patella and the opposite femoral cartilage. Labeling of the patellar cartilage becomes more pronounced in the later sections, whereas the reverse applies to the opposite cartilage.

Labeling remained present especially at the insertion sites of the ligaments, as shown for a collateral and an intraarticular ligament (Figure 7).

Antigen Retention, Local Inflammation, and Tissue Damage

Histologic examination of the ligaments at Days 14 and 28 after arthritis induction revealed no signs of nearby inflammation or tissue damage at sites of long-term antigen retention (see above). At Days 14 and 28 the presence of synovial exudate cells in the joint cavity diminished, enabling examination of possible attachment of inflammatory cells to antigen-containing cartilage structures. Preferential attachment of granulocytes or mononuclear cells at sites with local antigen retention was not observed. Earlier observations revealed that immune arthritis induces local chondrocyte death.[9] Areas with dead chondrocytes were sometimes found associated with retained

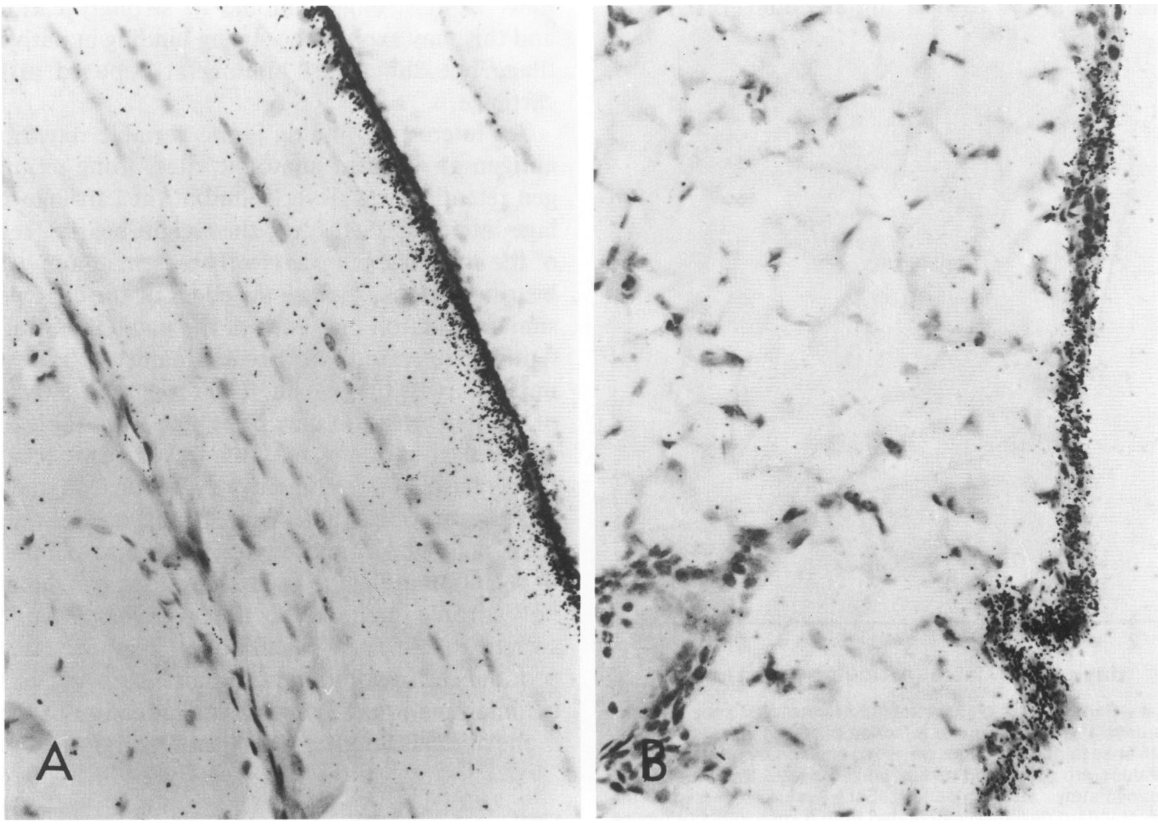

Figure 3—Autoradiographs of a collateral ligament (*A*) and synovial tissue (*B*) at Day 7 after intraarticular injection of ^{125}I-mBSA in nonimmune mice. Labeling is present at the whole surface of the ligament and at the synovial lining cells. Note the absence of inflammation. (H&E, ×206)

antigen, but the opposite situation, ie, antigen retention without nearby chondrocyte death or just antigen absence in areas with chondrocyte death, was most frequently observed.

Discussion

Our data indicate that in immune mice there is rapid disappearance of IA antigen from the synovial exudate and synovial lining and somewhat retarded clearance of antigen trapped in fibrinlike material in the joint cavity. Long-term antigen retention was found in avascular and hypovascular structures of the joint, albeit not along the whole surface. The extent of elimination and retention seems to be modulated by the presence and severity of joint inflammation. Antigen persistence was not anatomically related either to local inflammation or to local tissue damage.

The overall picture of antigen localization after IA antigen injection is the result of forces leading to its retention, predominantly at avascular and hypovascular structures, and forces aimed at elimination of the antigen. Shortly after intraarticular injection in immune animals, antigen is found in the synovial exudate and the synovial lining cells but not in the synovium, probably indicating extremely rapid antigen clearance from this tissue. Elimination of mBSA from the joint cavity is enhanced by the presence of inflammatory cells, which phagocytose and remove the antigenic material. Our observations in three experimental groups, ie, nonimmune animals in which antigen did not cause inflammation, immune animals in which antigen caused arthritis, and immune animals with an already existing arthritis in which antigen injected for a second time caused extremely severe arthritis, indicated most rapid elimination of antigen in the last group, having the most severe joint inflammation, whereas antigen clearance was slowest from the noninflamed joints of the first group.

The lesser sensitivity of the immunofluorescence method, compared with autoradiography in demonstrating antigen in the cartilage surface, may be caused by steric hindrance of the antiserums by matrix proteoglycans. In the immune animals detection of antigen with fluorescent antiserums may be further hampered by shielding of antigen already complexed with mouse anti-mBSA antibodies.

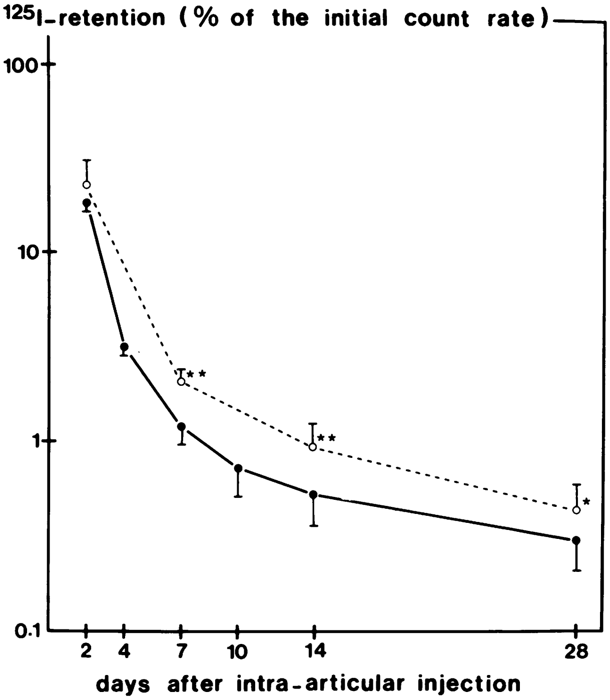

Figure 4—Radioactivity measurements of dissected knee joints at various days after intraarticular injection of 100 μg ^{125}I-mBSA (1 μCi) into both knee joints of immune (●——●) and nonimmune (O - - - O) mice. Values are expressed as the percentage of the initial count rate immediately after injection. Each value represents the mean ± standard deviation calculated from 6 knee joints of groups of 3 mice. Significant differences determined by the Wilcoxon test are indicated as follows: *$P < 0.05$; **$P < 0.02$.

Long-term retention of mBSA at avascular and hypovascular structures of the mouse joint may be determined by several factors. Both *in vitro* and *in vivo* experiments[12,13] have shown that antibodies are able to penetrate the superficial layers of cartilage, permitting antigen to be trapped by immune complex formation within the cartilage. Our immunofluorescence data indicate that in immune mice this mechanism is operative. However, it seems likely from our studies that antibody-mediated trapping is not the sole determinant of long-term retention. The physicochemical properties of mBSA seem to be important, because kinetics of this antigen differ profoundly from those of the native serum protein. BSA is eliminated more rapidly from the knee joints of nonimmune rabbits than from those of immune rabbits,[14] whereas the reverse applies to mBSA in mice. The importance of nonimmune binding of mBSA to avascular articular surfaces is furthermore suggested by the relatively weak binding of injected antigen at cartilage surfaces of already chronically inflamed joints. Cartilage in these joints is depleted of proteoglycans,[9] and this will lead to a diminished negative fixed charge density.[15] Methylation changes serum albumins from strongly anionic to strongly cationic,[16] and this may explain the strong binding at native cartilage but diminished binding at depleted arthritic cartilage.

An interesting finding is the variable retention of antigen at different anatomic sites. Long-term antigen retention was never found at the cartilage–cartilage interaction sites, eg, the weight-bearing regions of the condyles, but was clearly present at non-weight-bearing regions, such as the edges of the condyles. A similar situation may exist at the patellofemoral site. Antigen retention was predominantly found at the upper part of the patella. The topographic variation of antigen retention may be related to variable physicochemical properties of the cartilage, mechanical forces pushing the antigen away, or local degradation of antigen, for example, by neutral proteases released from activated chondrocytes.[17] Antigen elimination or retention at ligaments is determined by whether or not chronic granulation tissue develops on these structures. Antigen retention at ligaments is most pronounced in nonimmune animals showing no signs of inflammation. After arthritis induction in the immune animals long-term antigen retention was found

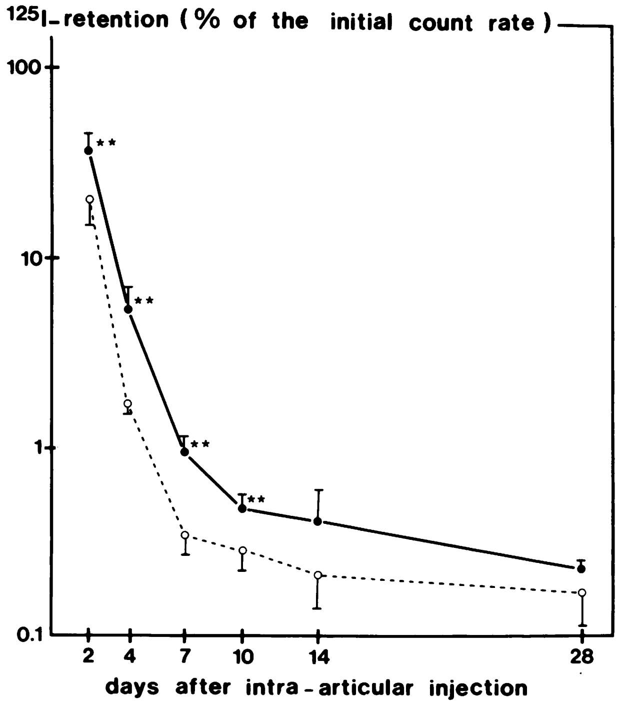

Figure 5—Radioactivity measurements of dissected knee joints at various days after intraarticular injection of 100 μg ^{125}I-mBSA (1 μCi) into the right (O - - - O) and left (●——●) knee joints of immune mice with a 4-week mBSA-induced arthritis in the right knee joint. Values are expressed as the percentage of the initial count rate immediately after injection. Each value represents the mean ± standard deviation calculated from groups of 5 mice. Significant differences determined by the Wilcoxon test are indicated as follows: *$P < 0.05$; **$P < 0.02$.

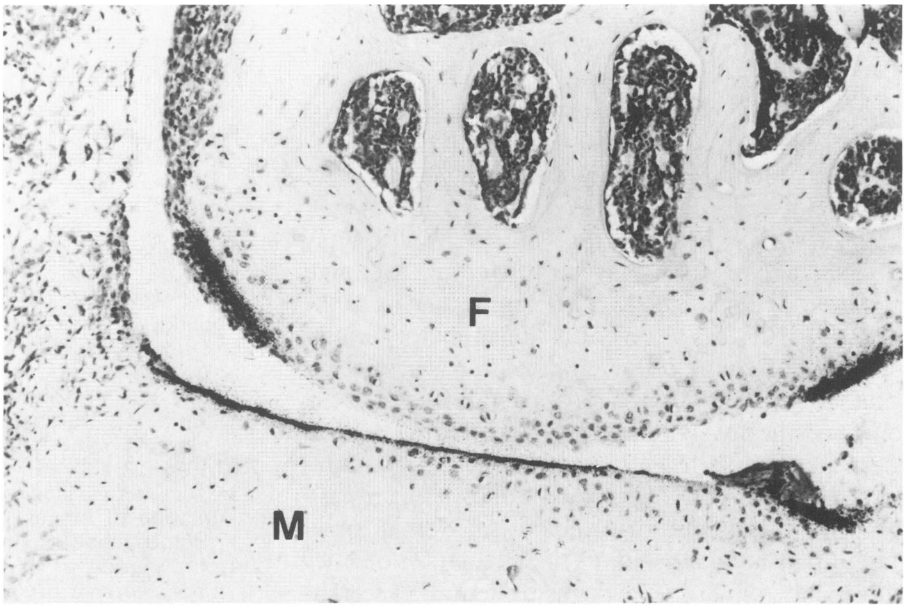

Figure 6—Autoradiograph of a femoral condyle (*F*), which shows persistence of labeling at the edges and absence of labeling in the central region. Note also the labeling of the meniscal cartilage (*M*). (H&E, ×83)

at the insertion sites that remained free of nearby inflammation but not at the sites with pannuslike overgrowth.

The consequences of maintaining small quantities of antigen in immune animals for long periods of time are not clear. Retention in the joint has been postulated as a mechanism underlying the apparent chronicity of inflammation after a single intraarticular injection in immune animals. A prerequisite for this concept is that the retained material is still immu-

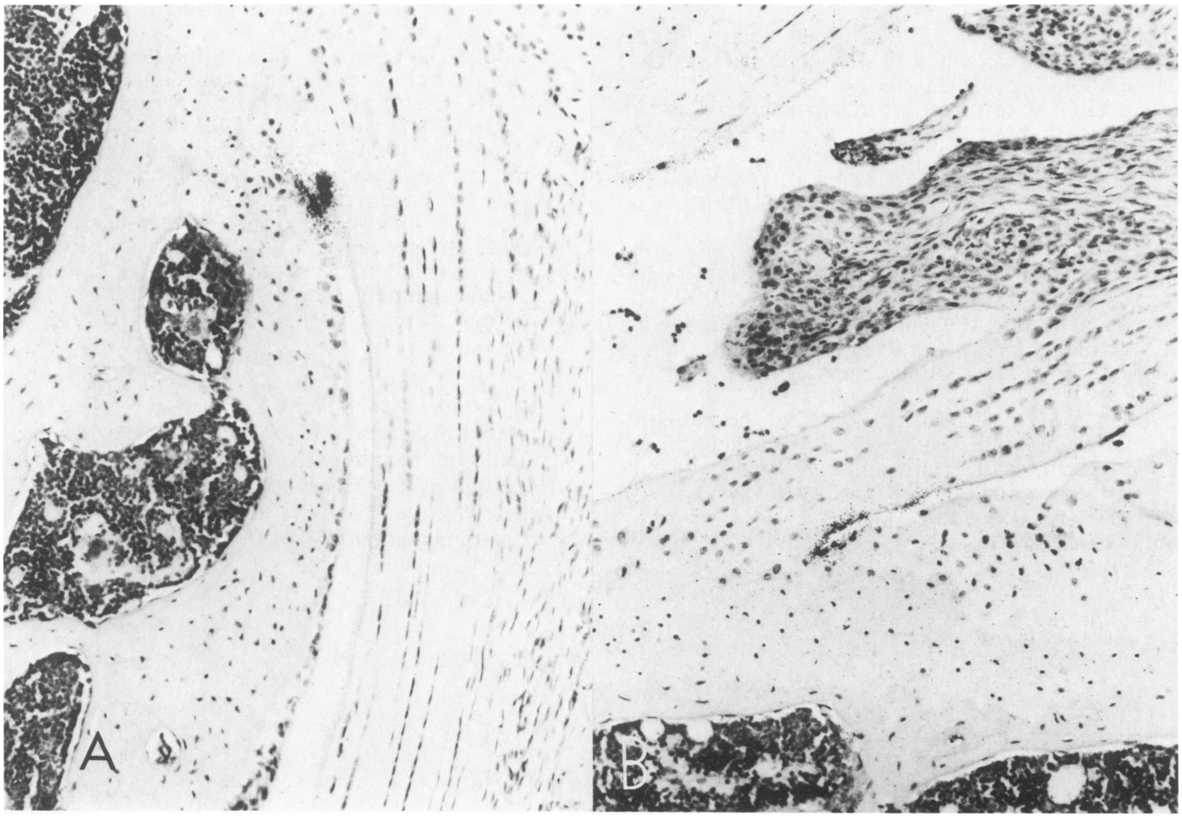

Figure 7—Autoradiograph of a collateral (*A*) and intraarticular (*B*) ligament at Day 28 after intraarticular injection of ^{125}I-mBSA in immune mice. Labeling is still present at the insertion sites. (H&E, ×83)

noreactive antigen. This was demonstrated for BSA retained in arthritic rabbit knee joints[18,19] and also for HSA retained at immunization sites in mouse footpads.[20] Our data indicate that antigenic material is retained at special sites of the avascular and hypovascular structures of the joint in immune mice, but it does not cause inflammation or tissue damage at these sites. One possibility relevant to the chronicity of joint inflammation may be the leakage of small amounts of this antigen to other compartments of the joint, where it does behave as an inflammatory stimulus, thus sustaining a low grade of chronic joint inflammation. In addition, this antigen may render the joint specifically hypersensitive to antigen. Such a phenomenon was recently demonstrated at immunization sites in mouse footpads,[20] and recent experiments in our laboratory demonstrated that small amounts of antigen given in the circulation of mice with unilateral chronic arthritis do induce exacerbation of the arthritis, whereas the contralateral knee joint does not react to this antigenic stimulus. Exacerbations due to local hypersensitivity to circulating antigens may play an important role in the chronicity of disease states like rheumatoid arthritis.

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